AWARD NUMBER: W81XWH-10-1-0504

TITLE: A New Therapeutic Strategy for Autosomal Dominant Polycystic Kidney Disease: Activation of AMP Kinase by Metformin

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REPORT DATE: July 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 July 2011	Annual	1 Jul 2010 – 30 Jun 2011

4. TITLE AND SUBTITLE

A New Therapeutic Strategy for Autosomal Dominant Polycystic Kidney Disease: Activation of AMP Kinase by Metformin

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0504

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University
New Haven, CT 06520

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Autosomal dominant polycystic kidney disease is a common inherited disorder. Patients are born with normal kidneys but, over the course of decades, they develop large fluid filled cysts that damage the normal kidney tissue. The damage caused by these cysts can lead ultimately to kidney failure, necessitating kidney transplantation or dialysis. There are currently no approved medications for this condition. Recent research reveals that the formation of cysts is due in part both to inappropriate cell growth and fluid secretion. The enzyme AMPK controls a number of cellular pathways, including those involved in cell growth and fluid secretion. Drugs that activate AMPK, therefore, may constitute an effective therapeutic option for slowing or preventing cyst growth. This research project is aimed at examining the potential of an approved, widely used, inexpensive and low-toxicity drug that can activate AMPK as a potential therapy for the treatment of polycystic kidney disease.

15. SUBJECT TERMS
Autosomal Dominant Polycystic Kidney Disease; Metformin

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	23	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by slow and continuous development of cysts derived from renal tubular epithelial cells. The cysts profoundly alter renal architecture, compressing normal parenchyma and compromising renal function. Nearly half of ADPKD patients ultimately require renal replacement therapy. ADPKD is a common genetic disorder, affecting at least 1 in 1,000 individuals (*1*). There are currently no effective specific clinical therapies for ADPKD. Cystic growth and expansion in ADPKD are thought to result from both fluid secretion into cyst lumens and abnormal proliferation of the cyst-lining epithelium. The rate of fluid secretion into the cyst lumen is directly proportional to the amount of the Cystic Fibrosis Transmembrane Regulator (CFTR) chloride channel in the apical membranes of cyst-lining epithelial cells (*2*). The cells surrounding the cysts manifest increased proliferation (*3, 4*). Mammalian Target of Rapamycin (mTOR) activity is elevated in models of PKD and is likely to be responsible, at least in part, for this hyperproliferative phenotype (*3*). mTOR is a serine/threonine kinase that regulates cell growth and proliferation, as well as transcription and protein synthesis. Interestingly, both the CFTR chloride channel and the mTOR signaling pathway are negatively regulated by the "energy sensing" molecule, Adenosine Monophosphate-activated Protein Kinase (AMPK). AMPK phosphorylates and directly inhibits CFTR, and indirectly antagonizes mTOR through phosphorylation of TSC2 and Raptor (*5-8*). Both of these actions are consistent with the role of AMPK as a regulator that decreases energy-consuming processes such as transport, secretion, and growth when cellular ATP levels are low (*9*). Thus, a drug that activates AMPK might inhibit both the secretory and the proliferative components of cyst expansion. Metformin, a drug in wide clinical use for both non-insulin dependent diabetes mellitus and Polycystic Ovary Syndrome, stimulates AMPK (*10, 11*). We therefore wish to examine whether metformin-induced activation of AMPK can slow cystogenesis through inhibition of mTOR-mediated cellular proliferation and inhibition of CFTR-mediated fluid secretion.

Body

Research Accomplishments Associated with Each Task Outlined in the Approved Statement of Work

Task 1. Characterize the effects of AMPK stimulation on its downstream targets in renal epithelia *in vivo* (months 1-20). The studies encompassed in this task group are designed to determine whether and how AMPK stimulation impacts upon the cellular pathways that are involved in cyst development and expansion. These experiments will make use of cultured MDCK cells and in vitro assays of both AMPK activation and the functional status of AMPK's down-stream targets.

Task 1a. **Establish AMPK stimulation by its pharmacologic activator, metformin in renal epithelial cells *in vitro*.** By immunoblotting for the downstream targets of AMPK, we will confirm that AMPK stimulation has the same effects on its downstream targets in renal epithelia as has been reported in other tissues **(months 1-8)**.

Research Accomplishments: Metformin stimulates AMPK and pACC.

We first treated Madin Darby Canine Kidney (MDCK) renal epithelial cells with metformin to evaluate AMPK activation. Activated AMPK is phosphorylated at residue Thr172 of its α subunit. We performed Western blotting using a phosphospecific antibody to measure the level of the phosphorylated AMPK, pAMPK (Fig. 1a). We found that incubation with metformin for as little as 2 hours significantly increases pAMPK levels (Fig. 1b). To determine whether this effect was correlated with increased phosphorylation of an AMPK target, we evaluated metformin's effect on the AMPK-mediated inhibitory

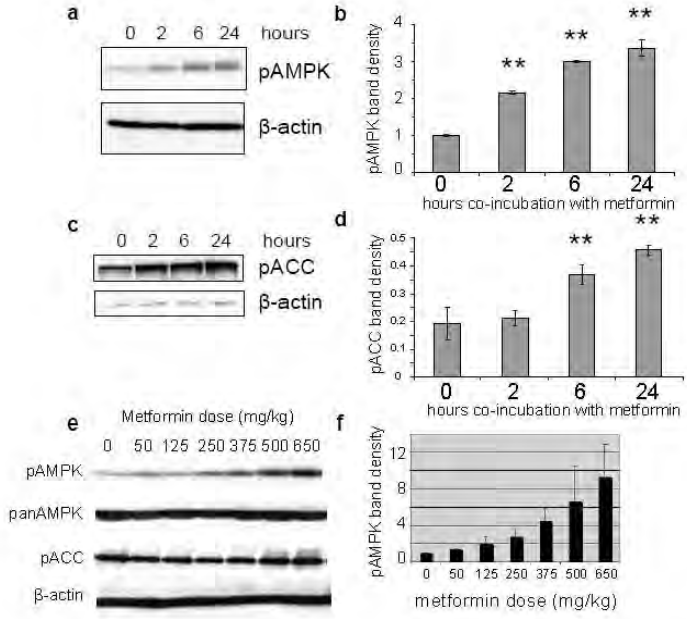

Figure 1: Metformin activates AMPK in vitro and in vivo. (a) MDCK cells were incubated with 1.0 mM metformin for the number of hours stated. Cells lysates blotted for pAMPK, the activated form of AMPK. (b) Quantitation of pAMPK band density normalized to β-actin. Comparisons of the mean (± SEM) are shown for each timepoint. (c) MDCK cells treated as above, blotted with pACC, a downstream target of pAMPK. (d) Comparisons of the mean band density relative to β-actin (± SEM) are shown for each timepoint. (e) 8-week-old C57BL/6 mice were treated with intraperitoneal metformin or with vehicle for three. Western blot analysis of kidney homogenates using anti-pAMPK demonstrates increasing activation of AMPK with increasing metformin dosing. (f) Quantitation of Western blot of in vivo pAMPK levels by normalized band density to β-actin.

phosphorylation of Acetyl-CoA Carboxylase (ACC) (Fig. 1c). Incubation of MDCK cells with metformin produced a significant increase in pACC levels in six hours (Fig. 1d). In AMPK α1 knockdown cells, metformin's effects on pAMPK and pACC levels are substantially blunted. Treatment of mice with increasing doses of metformin, administered daily for three days, results in increasing levels of pAMPK throughout the nephron (Fig. 1e and 1f).

Task 1b. **Characterize the physiological consequences of inhibition of AMPK downstream target CFTR.** This will be accomplished via short circuit current measurements performed on MDCK cells transfected to express CFTR and grown on permeable filter supports **(months 6-16)**.

Research Accomplishments: Inhibition of CFTR-dependent Isc by Metformin in MDCK Cells is AMPK-dependent.

We next examined the effect of metformin treatment on the CFTR chloride channel, which is inhibited by AMPK phosphorylation (*12-14*). Since CFTR drives, at least part of the fluid secretion

in PKD cystogenesis, we hypothesized that metformin-stimulated AMPK activity would inhibit CFTR channels in renal epithelial cells and slow the rate of cyst growth (*15, 16*). To test whether metformin inhibits CFTR via AMPK in a kidney-derived epithelial cell line, CFTR was expressed by adenoviral transduction in three different polarized MDCK type II cell lines stably transfected with either an empty vector, or shRNA plasmids directed against two isoforms of the catalytic α subunit of AMPK. MDCK cells endogenously express high concentrations of the α1 isoform of the AMPK catalytic α subunit and very low concentrations of the α2 isoform. Expression of the α1 shRNA construct reduced this protein's expression by ~90%, whereas the α2 shRNA had no effect on α1 protein expression. Knockdown of α1 also reduced the level of total phospho-AMPK

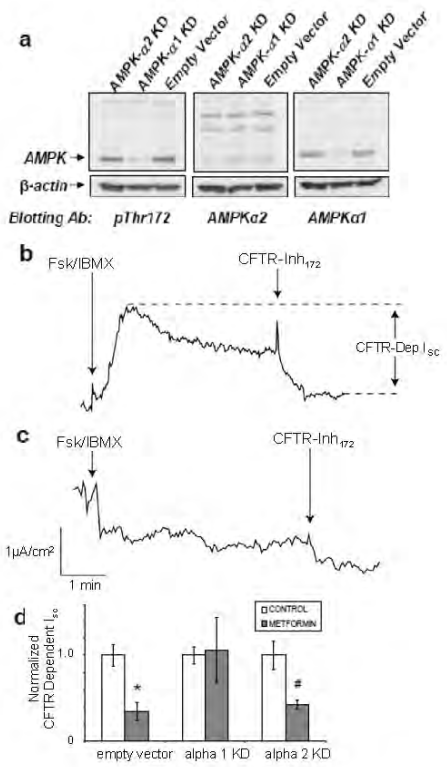

Figure 2: Metformin inhibits Isc in an AMPK-dependent manner. (a) MDCK cells stably expressing either empty vector or shRNA plasmids directed against either the catalytic α1 or α2 subunits of AMPK (AMPK-α1 KD and AMPK-α2 KD cells, respectively) were blotted with antibodies against either pThr172, AMPKα2, or AMPKα1 to measure the level of AMPK expression. (b) A representative Isc trace of cells with or without 1 mM metformin pre-treatment. Mock-transduced or NH2-terminally GFP-tagged-CFTR-transduced MDCK "empty vector" control cells, AMPK-α1 KD or AMPK-α2 KD cells were treated with 1 mM metformin or vehicle for 2-4 h prior to Ussing chamber measurements of Isc. A representative Isc trace of vehicle pre-treated CFTR-expressing "empty vector" control MDCK cells treated with IBMX and forskolin and then CFTR-Inh172 at the indicated times is shown. (c) A similar representative trace of mock-transduced "empty vector" control cells shows no response to these cAMP agonists or to CFTR-Inh172. There was also no significant change in Isc following addition of 10 μM amiloride, indicating that the epithelial Na+ channel does not significantly contribute to Isc in these MDCK cells. (d) Comparisons of the normalized mean (± SEM) CFTR-dependent Isc in "empty vector" control, AMPK-α1 KD, and AMPK-α2 KD cells with (dark gray bars) or without (white bars) metformin pre-treatment.

(pAMPK) by ~90% (Fig. 2a). CFTR-dependent Isc was measured for cells grown on filters mounted in Ussing chambers for four days following adenoviral transduction, with or without exposure to 1 mM metformin for 2-4 hours prior to measurement. To initiate CFTR-mediated secretion, CFTR-expressing and mock-transduced MDCK cells were treated with the cAMP agonists IBMX and forskolin, and the experiment was concluded through application of the specific CFTR inhibitor CFTR-Inh172 (*17*). Typical traces of Isc changes are shown in Fig. 2b and Fig. 2c. In CFTR-expressing cells there was generally an early peak in Isc within 1-2 min following forskolin/IBMX treatment, followed by a lower plateau current within ~5 min. This remaining current was sensitive to inhibition by CFTR-Inh172. Metformin (1 mM) pretreatment of empty vector-transfected and AMPK-α2 knockdown MDCK cells significantly reduced CFTR-dependent Isc by 60-70% relative to cells pretreated with vehicle (Fig. 2d). However, there was no metformin-dependent inhibition of CFTR current in AMPK-α1 knockdown MDCK cells, suggesting that the metformin-induced inhibition of CFTR occurs specifically via an AMPK-α1-dependent mechanism.

Task 1c. **Assess AMPK-mediated mTOR inhibition.** This will be accomplished both by direct assay of the phosphorylation status of downstream mTOR targets and by determining the physiological implications of this inhibition through measurements of cell proliferation **(months 10-20)**.

Research Accomplishments:

Inhibition of mTOR by Metformin in MDCK Cells is AMPK-dependent.

To determine whether metformin induces AMPK-mediated inhibition of mTOR activity, we tested whether mTOR activity is diminished in MDCK cells cultured in the presence of metformin by blotting for the phosphorylated form of the mTOR downstream target ribosomal S6K p70 subunit (p70 S6K) (Fig. 3a) relative to pan-S6K (Fig. 3b). This inhibition is time-dependent, with increasing exposure to metformin resulting in greater suppression of this pathway. Total S6K levels remain constant. The inhibition takes longer to achieve than inhibition of CFTR or ACC,

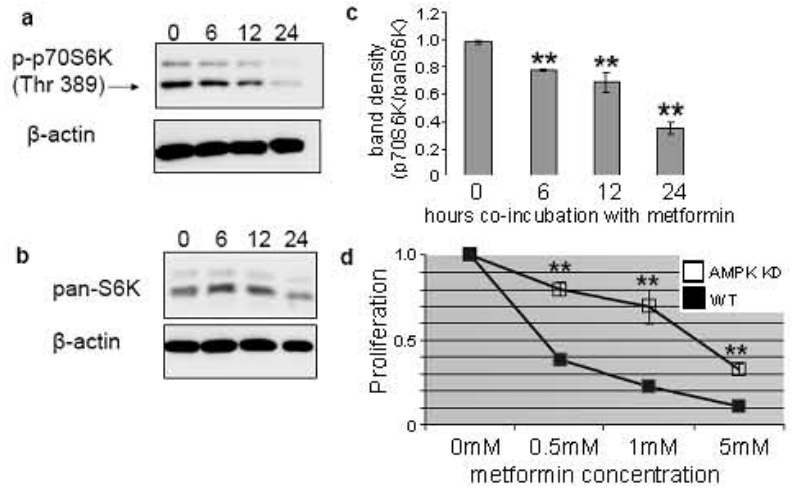

Figure 3: Metformin inhibits phosphorylation of the mTOR downstream target, p70 S6 Kinase, and slows cellular proliferation in an AMPK-dependent manner. (a) A subconfluent monolayer of MDCK cells was incubated with 1.0 mM metformin for the indicated time. Cells lysates were blotted for the downstream marker of mTOR activity (a) p70 S6K and (b) total S6K. (c) Quantitation of phospho-S6K Western blot band density normalized to β-actin. Comparisons of the mean (± SEM) are shown for each timepoint. (d) Effect of metformin on proliferation of control MDCK cells and MDCK cells stably transfected with shRNA against AMPK, graphed relative to control. The y-axis represents cell number at each concentration of metformin, normalized to the control value measured for the same cell type at the same time point without metformin treatment.

consistent with the fact that AMPK indirectly inhibits mTOR via TSC2/1 and Rheb (Fig. 3c). This effect is markedly less pronounced in AMPK-α1 KD cells. To evaluate whether these changes in phospho-protein levels translated into changes in proliferation, an Alamar Blue assay was used to quantitate proliferation in wild-type and AMPK-α1 knockdown MDCK cells. The y-axis depicts cell number measured at each given concentration of metformin and normalized to the control value, which was obtained for the same cell type at the same time point without metformin treatment. Wild-type MDCK cells exhibited a metformin dose-dependent decrease in proliferation, while this response was significantly diminished in the AMPK-α1 knockdown MDCK cells. (Fig. 3d) It should be noted that at the highest concentration of metformin tested (5 mM) substantial growth suppression was detected in AMPK knockdown cells. This may be due to the low level of residual AMPK that is expressed in these knockdown cells (see Fig. 2a) or to effects of high doses of metformin on yet to be identified AMPK-independent pathways. A similar suppressive effect of metformin treatment on proliferation is observed in vivo. We performed immunofluorescence analyses on kidneys from metformin treated and vehicle treated cystic Pkd1flox/-;Ksp-Cre mice using an antibody directed against Ki67, a marker of actively proliferating cells. In kidneys from vehicle-treated mice 19.7±3.8% s.e.m. of the cells exhibited Ki67 positivity (450 cells counted from each of n=6 mice) in comparison to 10.6±3.6% s.e.m. (450 cells counted from each of n=4 mice) in metformin-treated mice ($p<0.0074$). To assess whether the effects of metformin treatment on proliferation correlate with the level of mTOR activity in the cystic kidneys before and after metformin treatment, we performed immunohistochemistry using an antibody directed against the activated form of an mTOR target. We stained tissue from control and metformin treated cystic mice with an antibody that detects the phosphorylated form of 4E-BP1 (), an mTOR target whose level of phosphorylation is commonly used to report on levels of mTOR activity (*18*). We find that the level of p4E-BP1 is generallyhigher in the cyst lining epithelial cells in control animals as compared to those observed in metformin treated animals, consistent with the interpretation that metformin treatment reduced the level of mTOR activation.

Planned Studies for Task 1: We will assess the extent to which varied dosing regimens can produce AMPK activation in wild type mice. In addition, we will determine whether activation of AMPK by metformin treatment exhibits any isoform preference. To test this possibility, we will measure activation of renal AMPK by metformin using antibodies that will allow us to distinguish between the α1 or α2 isoforms of the AMPK α-subunit. In addition, we will assess the effects of AMPK activation on the distribution and activity of renal CFTR in vivo in mice treated with metformin. Finally, we will determine whether the metformin dosing regimen that is optimal for achieving AMPK activation is also optimal for producing mTOR inhibition by using antibodies directed against p4E-BP1 to assess the level of mTOR activation in kidney tissue derived from treated animals.

Task 2. Evaluate the *in vitro* and *in vivo* effects of metformin-induced AMPK inhibition of mTOR and CFTR in the context of *in vitro* and *in vivo* models of cystic kidney disease (months 12-36).

***Task 2a*. Determine the effect of metformin treatment on average cyst size and cyst number using *in vitro* models of cystogenesis.** These experiments will be performed with renal epithelial cells that spontaneously form cysts when suspended in a collagen matrix. Cyst size and number will be determined by quantitative fluorescence microscopy techniques **(months 12-24)**.

Research Accomplishments: Metformin treatment slows cystogenesis *ex vivo*.

Two-dimensional culture models do not accurately depict cell growth in the three-dimensional environment in which cysts develop. To evaluate metformin's effects in the context of cystogenesis, we suspended MDCK cells in a three-dimensional collagen matrix and allowed them to form cysts spontaneously in the presence of forskolin and IBMX (*19*). Cultures co-incubated with metformin for the duration of cyst growth produced significantly smaller cysts than those similarly treated with forskolin and IBMX alone (p=0.003, unpaired t-test, n=3 gels for each experimental condition) (Fig 4a).

We next tested the effect of metformin on ex vivo cystogenesis. Embryonic kidneys (E12.5) were removed from C57/B6 mice. One embryonic kidney was cultured in the presence of membrane permeable 8-Br-cAMP to stimulate fluid secretion, while the contralateral kidney was co-incubated with 8-Br-cAMP and metformin for 4 days. Culture in the presence of 8-Br-cAMP induces cyst formation in embryonic mouse kidneys (*20*). Metformin treatment significantly decreased fractional cyst area (p=0.04, unpaired t-test with n=4 for each experimental condition). On day 5, metformin was removed from the treated embryonic kidney and cyst growth recommenced in the

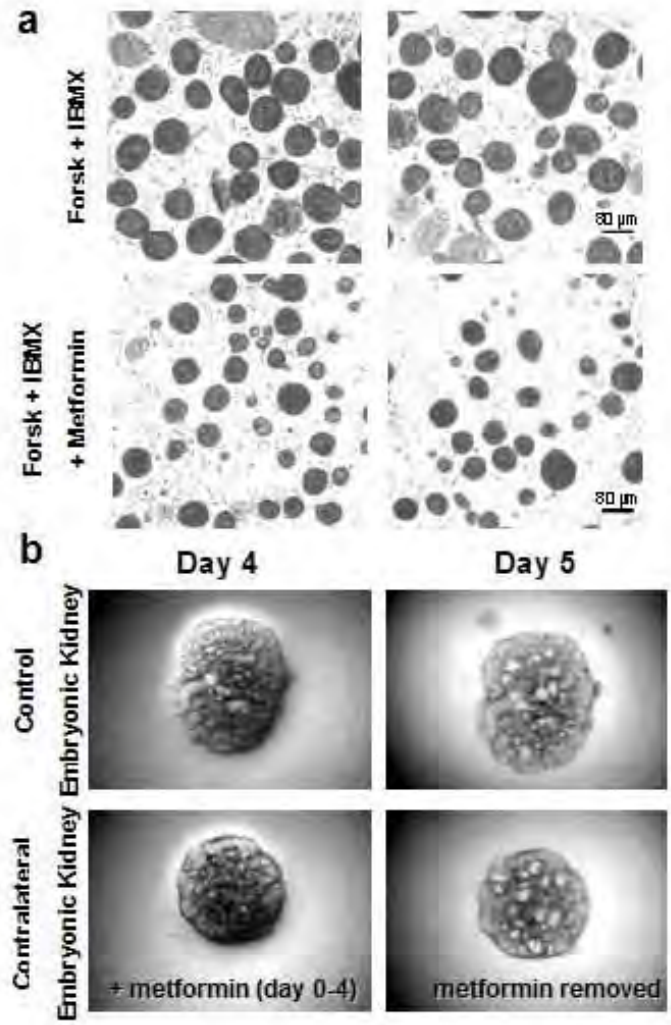

Figure 4: **Metformin reduces cyst size *in vitro* and *ex vivo*.** (a) Representative light micrographs of MDCK cell cysts grown in collagen gels. Cysts were treated with forksolin and IBMX, to enhance apical fluid secretion, with (bottom) or without (top) 1.0 mM metformin for 20 days. Gels were melted and the cysts were allowed to precipitate to the bottom for imaging. (b) Metformin treatment reduces cyst size in an *ex vivo* model of renal cystogenesis. Embryonic kidneys were placed in culture at E12 and maintained for 5 days in the continued presence of 100 µM 8-Br-cAMP. Representative light microscopic images are shown from one mouse. Each row shows the same kidney. The contralateral kidney (bottom row) was treated with metformin for 4 days, and then switched to normal media, illustrating that the embryonic kidney remains viable and capable of cystogenesis.

treated kidney, demonstrating that metformin treatment slowed cyst growth without affecting the viability of the tissue (Fig. 4b).

Task 2b. **Perform an *in vivo* trial of metformin treatment on Ksp-Cre, Pkd1$^{flox/-}$ mice.** This mouse model represents a very severe model of ADPKD. These experiments will permit the potential for metformin therapy to slow disease progression to be evaluated in mice that have developed cystic disease prior to the initiation of treatment **(months 12-28)**. These experiments will utilize 75 mice, 25 of which will be used in months 1-12 for breeding purposes, to generate a stable colony of 50 mice with the required genotypes that will be used in the experiments associated with this task.

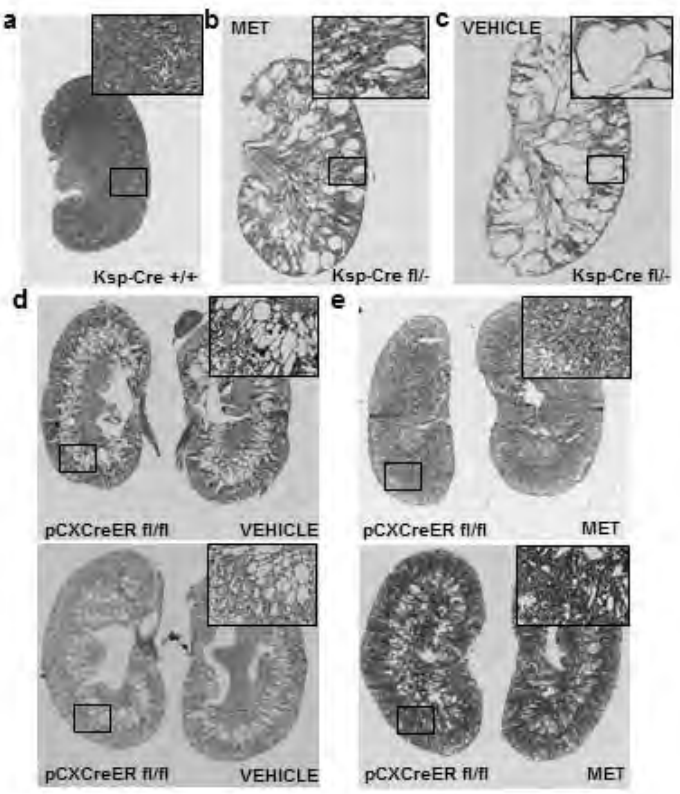

Figure 5: **Metformin treatment reduces cystic index in two mouse models of ADPKD.** Representative mid-sagittal sections from the kidneys of a (a) *PKD1$^{+/+}$;Ksp-Cre* mouse, (b) metformin-treated *PKD1$^{flox/-}$;Ksp-Cre* mouse, and (c) vehicle-treated P*KD1$^{flox/-}$;Ksp-Cr*e mouse at P7. The metformin and vehicle treated mice were given daily weight-adjusted intraperitoneal injections from P4 until P6. Representative images from *PKD$^{flox/-}$;pCX-CreER* mice treated with vehicle (d) or metformin (e) from P7-P17, with Cre induction at P9-10.

Research Accomplishments: Metformin treatment slows cystogenesis in an aggressive constitutive *in vivo* model of PKD.

We next tested whether metformin slows cyst growth in a murine model of PKD. Initially, we used the most aggressive viable murine model of PKD (Pkd1flox/-;Ksp-Cre) in which there is progression of renal cystic disease within the first week of life and death between two and three weeks of life (*4*). We treated these mice with daily intraperitoneal injections of metformin (300 mg/kg/day) dissolved in a 5% dextrose solution from P4 until P6. This is a dose known to activate AMPK (*21*). Mice were then sacrificed and kidneys harvested at P7. The vehicle treated Pkd1flox/;Ksp-Cre kidneys (Fig. 5c) were profoundly cystic and greatly enlarged compared to the Pkd1+/+;Ksp-Cre kidneys (Fig. 5a). In contrast cyst, burden is significantly reduced in the kidneys from the metformin-treated Pkd1flox/-;Ksp-Cre mice (Fig. 5b). Because metformin can affect body weight, kidney weight: body weight ratio was not used as an endpoint (*22*). Instead, the effect of metformin on renal morphology was quantitated by evaluating cystic index, which determines the fraction of a given section that corresponds to luminal area (including both tubule and cyst lumens). Untreated

Pkd1flox/-;Ksp-Cre kidneys had a cystic index of 71.4±4.0% s.e.m., whereas that of metformin treated Pkd1flox/-;Ksp-Cre kidneys was 51.8±5.2% s.e.m. (p=0.029; unpaired t-test with n=4 control and n=8 metformin treated mice). In wild type kidneys, this evaluation calculates a cystic index of 10% due to tubular lumens. Notably, while the metformin-treated kidney is still cystic, it displays significantly more parenchyma than the vector-treated control. While metformin might prevent further cyst growth, it is unlikely that treatment reduces the size of pre-existing cysts.

Task 2c. **Evaluate the effect of metformin in a mouse model of inducible PKD.** We will use an inducible mouse model of PKD (pCAGGS-cre, tamoxifen-activatable $Pkd1^{flox/-}$ mice), which will allow mice to be pre-treated with metformin prior to disease induction. This will allow us to gauge the therapeutic benefit of metformin under conditions that may more accurately mimic at least certain aspects of the progression of the human disease **(months 20-36)**. These experiments will utilize 75 mice, 25 of which will be used in months 1-12 for breeding purposes, to generate a stable colony of 50 mice with the required genotypes that will be used in the experiments associated with this task.

Research Accomplishments: Metformin treatment slows cystogenesis in an inducible *in vivo* model of PKD.

We established an inducible model for Pkd1 inactivation using a conditional Pkd1flox allele in combination with a tamoxifen inducible Cre recombinase (pCX-CreERTM) (*4, 23, 24*). Induction of Cre expression prior to P13 leads to rapidly progressive cystic disease in Pkd1flox/flox animals (*25*). In this system, it is possible to initiate metformin treatment prior to or during cyst development. Thus, this model might more accurately replicate the clinical scenario, in which metformin therapy could commence early in the disease process and act to prevent or slow subsequent cyst development. We initiated metformin treatment (300 mg/kg/day) at day P7 and then injected intraperitoneal tamoxifen at day P9 and P10 to initiate disease induction. We continued daily metformin injections until P18, when the animal was sacrificed and kidneys harvested for histology and cystic index evaluation. Once again, metformin treatment resulted in a smaller fractional cyst burden than vehicle-treated controls. The kidneys from vehicle-treated mice had a cystic index of 43%, whereas that of metformin treated mice was 31% (p=0.041, unpaired t-test with n=6 for vehicle treated and n=7 for metformin treated mice), a decrease of nearly one-third in the cyst burden (Fig. 5d,e).

Planned Studies for Task 2: The studies outlined in the Planned Studies for Task 1 will identify a metformin dosing regimen that is optimal for achieving activation of renal AMPK. We will test the efficacy of this regiment in preventing or slowing cystogenesis in two mouse models of autosomal dominant polycystic kidney disease. We will cross the mice carrying a $Pkd1^{flox/-}$ genotype with mice expressing Cre under the Ksp-cadherin or the Pkhd1 promoters to generate rapidly and slowly progressive models of the disease, respectively. To assess the effects of optimal metformin treatment on cystogenesis in these animals we will measure kidney weight to body weight ratios, cystic indices, and plasma levels of BUN and creatinine. We are initiating a collaboration with an investigator who has generated conditional knockout mouse models of AMPK α1 and α2 isoform expression. When these models become available (and once approval has been granted for the relevant modifications of the ACURO animal use appendix and our IACUC animal use protocol) we will cross the AMPK α1 and α2 null alleles into homozygous and heterozygous $Pkd1^{flox/-}$ backgrounds. We anticipate that absence of expression of one or the other AMPK subunits exacerbates the progression of the disease and reduces the efficacy of metformin treatment.

Key Research Accomplishments

- Metformin stimulates AMPK and pACC.
- Inhibition of CFTR-dependent I_{sc} by Metformin in MDCK Cells is AMPK-dependent.
- Inhibition of mTOR by Metformin in MDCK Cells is AMPK-dependent.
- Metformin treatment slows cystogenesis ex vivo and in vivo.
- Metformin treatment slows cystogenesis in in vivo models of PKD.

Reportable Outcomes

- Peer reviewed primary data publication:

  Takiar, V., S. Nishio, J.D. King Jr., H. Li, L. Zhang, A. Karihaloo, K.R. Hallows, S. Somlo, M.J. Caplan. Activating AMPK slows renal cystogenesis. Proc. Nat. Acad. Sci, 108:2462-2467, 2011.

- Invited review articles:

  Takiar, V. and M.J. Caplan. Telling kidneys to cease and decyst. Nature Medicine, 16:751-752, 2010.

  Takiar, V. and M.J. Caplan. Polycystic Kidney Disease: Pathogenesis and Potential Therapies. Biochim. Biophys. ACTA, In press, 2011.

Conclusion

AMPK activity can be pharmacologically targeted with metformin to reduce the growth of renal cysts. Metformin acts through AMPK to decrease both epithelial fluid secretion by directly inhibiting CFTR, and to decrease cellular proliferation by indirectly targeting mTOR. Metformin stimulates AMPK phosphorylation in cultured MDCK renal epithelial cells, and this phosphorylation correlates with increased AMPK activity, as evidenced by an increase in the level of the AMPK-mediated inhibitory phosphorylation of ACC. Metformin's inhibitory action on CFTR-mediated chloride transport is AMPK-dependent. Additionally, we show that metformin inhibition of mTOR translates into an AMPK-dependent inhibition of cell proliferation. Using both an in vitro model of MDCK cell cystogenesis as well as embryonic kidneys ex vivo, we demonstrate that metformin decreases cyst size, and fractional cyst area, respectively. Finally, we illustrate the potential therapeutic utility of metformin by testing it in two murine models of ADPKD, both of which are attributable to inactivation of the gene encoding polycystin-1.

In this study, only one dose known to activate AMPK in vivo was tested. When considered on a simple mg/kg basis, this dose appears considerably higher than the current maximum dose prescribed for patients with diabetes or Polycystic Ovary Syndrome. However, human equivalent dose extrapolation is more accurately calculated based on body surface area than on weight. When this calculation is performed for a 60 kg adult, the dose used in our mouse studies extrapolates to a daily dose of approximately 1500 mg (43), which falls well within the range in which metformin is safely used in humans. We have not tested the efficacy of lower doses or of alternative dosing regimens in these mouse models. It is likely, however, based on the established pharmacokinetics of metformin, that single daily dosing is suboptimal, and thus we are almost certainly not observing the maximal suppressive effects that metformin could potentially exert on the severity of cyst growth.(44) Support for this contention derives from the data presented in Figure 4c, since in the embryonic kidney model, cyst growth rapidly resumes shortly after removal of metformin from the culture media. Thus, short term intermittent metformin exposure may not be adequate to optimally suppress cyst development. It is quite possible that even lower doses administered more frequently might produce beneficial effects in the setting of polycystic kidney disease. It is important to note that our efforts to assess effects of metformin treatment on renal functional parameters such as serum concentrations of BUN and creatinine were inconclusive, due in part to a large degree of inter-individual variability. Further studies, perhaps employing more slowly progressive disease models, will be required to reduce this variance and to assess the extent to which metformin treatment can protect or improve renal function in the setting of polycystic kidney disease. In addition, subsequent development of metformin for this clinical application will require pharmacokinetic and pharmacodynamic studies designed to identify an ideal dosing regimen that achieves maximal activation of renal tubular AMPK.

So What?

Metformin is taken by millions of Americans each year. It is currently FDA-approved for the treatment of Non-Insulin Dependent Diabetes (Type II DM) and, intriguingly, for Polycystic Ovary Syndrome, a disease that shares a name similar to that of Polycystic Kidney Disease but whose pathogenesis is even less well understood. In fact, metformin is often considered first line therapy for the treatment of Type II DM, due to its relatively small side effect profile. Recent literature suggests that metformin's activation of AMPK may be due to its ability to prevent AMP breakdown, although the exact mechanisms of action of metformin in Polycystic Ovary Syndrome or in Type II DM remain largely unknown.(30) Recent reports also suggest that metformin may

exert an anti-neoplastic effect. It has been reported that metformin acts in a dose-dependent manner to inhibit the proliferation of breast cancer cells and that this effect can be blocked in the presence of small interfering RNA directed against AMPK.(31) This inhibition is also associated with a decrease in mTOR activation, suggesting that metformin's anti-proliferative effect is directed through the activation of AMPK, and consequent inhibition of mTOR.

There are numerous therapies for ADPKD in development or in clinical trials, including vasopressin receptor inhibitors, calcium sensing receptor inhibitors, CFTR-inhibitors, cell cycle inhibitors, and rapamycin.(4, 9, 39, 40) Each of these strategies targets one or the other of the key processes (proliferation and secretion) thought to be involved in the pathogenesis of PKD. By acting through AMPK, metformin may offer the significant advantage of blocking both (SI 4). Moreover, metformin is already FDA approved and generally well-tolerated. The most serious, albeit rare, side effect of metformin is lactic acidosis and, since metformin is cleared by the kidney, chronic renal disease has been considered to be a potential predisposing factor for this complication. However, metformin use could ideally be initiated at an early stage in ADPKD progression, prior to the development of substantial cyst burden and compromise of renal function, thus allowing for maximal preventive benefit and minimizing the potential for renal dysfunction to limit the safe use of the drug.(41, 42) Given the relatively late onset and slow progression of ADPKD it is conceivable that, even if metformin were to have only modest effects in delaying or slowing cyst development, it might significantly increase the time to the development of end stage renal disease and perhaps reduce the need for renal replacement therapy.

We find that metformin stimulates AMPK, resulting in inhibition of both CFTR and mTOR, and thereby, both epithelial secretion and proliferation, respectively. Our data suggest the possible utility of metformin as a therapy for ADPKD and that AMPK is a novel potential pharmacological target for ADPKD therapy. The large body of knowledge associated with metformin administration could conceivably facilitate the translation of these findings into clinical trials to test the proposition that metformin is a safe and promising approach that exploits AMPK activity to treat this challenging disease.

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Appendix

Reprint of:

Takiar, V., S. Nishio, J.D. King Jr., H. Li, L. Zhang, A. Karihaloo, K.R. Hallows, S. Somlo, M.J. Caplan. Activating AMPK slows renal cystogenesis. Proc. Nat. Acad. Sci, 108:2462-2467, 2011.

Activating AMP-activated protein kinase (AMPK) slows renal cystogenesis

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Edited* by Gerhard Giebisch, Yale University School of Medicine, New Haven, CT, and approved December 14, 2010 (received for review August 9, 2010)

Renal cyst development and expansion in autosomal dominant polycystic kidney disease (ADPKD) involves both fluid secretion and abnormal proliferation of cyst-lining epithelial cells. The chloride channel of the cystic fibrosis transmembrane conductance regulator (CFTR) participates in secretion of cyst fluid, and the mammalian target of rapamycin (mTOR) pathway may drive proliferation of cyst epithelial cells. CFTR and mTOR are both negatively regulated by AMP-activated protein kinase (AMPK). Metformin, a drug in wide clinical use, is a pharmacological activator of AMPK. We find that metformin stimulates AMPK, resulting in inhibition of both CFTR and the mTOR pathways. Metformin induces significant arrest of cystic growth in both in vitro and ex vivo models of renal cystogenesis. In addition, metformin administration produces a significant decrease in the cystic index in two mouse models of ADPKD. Our results suggest a possible role for AMPK activation in slowing renal cystogenesis as well as the potential for therapeutic application of metformin in the context of ADPKD.

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the slow and continuous development of cysts derived from renal tubular epithelial cells. The cysts profoundly alter renal architecture, compressing normal parenchyma and compromising renal function. Nearly half of ADPKD patients ultimately require renal replacement therapy. ADPKD is a common genetic disorder, affecting at least 1 in 1,000 individuals (1). There currently are no effective specific clinical therapies for ADPKD.

Cystic growth and expansion in ADPKD are thought to result from both fluid secretion into cyst lumens and abnormal proliferation of the cyst-lining epithelium. The rate of fluid secretion into the cyst lumen is directly proportional to the amount of the cystic fibrosis transmembrane regulator (CFTR) chloride channel in the apical membranes of cyst-lining epithelial cells (2). The evidence suggesting that CFTR acts as a significant contributor to cyst growth has inspired preclinical trials of CFTR inhibitors in cell and animal models of renal cystic disease (3, 4).

The cells surrounding the cysts manifest increased proliferation (5, 6). Mammalian target of rapamycin (mTOR) activity is elevated in models of polycystic kidney disease (PKD) and probably is responsible, at least in part, for this hyperproliferative phenotype (5). mTOR is a serine/threonine kinase that regulates cell growth and proliferation as well as transcription and protein synthesis. Rapamycin inhibits mTOR's kinase activity (7, 8). Indeed, treatment with rapamycin has been shown to improve parameters of renal cystic expansion in several animal models of ADPKD (5, 9).

Interestingly, both the CFTR chloride channel and the mTOR signaling pathway are negatively regulated by the "energy-sensing" molecule, AMP-activated protein kinase (AMPK). AMPK phosphorylates and directly inhibits CFTR and indirectly antagonizes mTOR through phosphorylation of tuberous sclerosis protein 2 (TSC2) and Raptor (10–13). Both of these actions are consistent with the role of AMPK as a regulator that decreases energy-consuming processes such as transport, secretion, and growth when cellular ATP levels are low (14). Thus, a drug that activates AMPK might inhibit both the secretory and the proliferative components of cyst expansion. Metformin, a drug in wide clinical use for both non–insulin-dependent diabetes mellitus (type 2 DM) and polycystic ovary syndrome, stimulates AMPK (15, 16). We therefore examined whether metformin-induced activation of AMPK slows cystogenesis through inhibition of mTOR-mediated cellular proliferation and inhibition of CFTR-mediated fluid secretion.

Results

Metformin Stimulates AMPK and Phosphorylated Acetyl-CoA Carboxylase. We first treated Madin–Darby canine kidney (MDCK) renal epithelial cells with metformin to evaluate AMPK activation. Activated AMPK is phosphorylated at residue Thr^{172} of its α subunit. We performed Western blotting using a phosphospecific antibody to measure the level of the phosphorylated AMPK (pAMPK) (Fig. 1*A*). We found that incubation with metformin for as little as 2 h significantly increases pAMPK levels (Fig. 1*B*). To determine whether this effect was correlated with increased phosphorylation of an AMPK target, we evaluated metformin's effect on the AMPK-mediated inhibitory phosphorylation of acetyl-CoA carboxylase (ACC) (Fig. 1*C*). Incubation of MDCK cells with metformin produced a significant increase in phosphorylated ACC (pACC) levels in 6 h (Fig. 1*D*). In AMPK-α1 knockdown (AMPK-α1–KD) cells, metformin's effects on pAMPK and pACC levels are substantially blunted (Fig. S1). Treatment of mice with increasing doses of metformin administered daily for 3 d results in increasing levels of pAMPK throughout the nephron (Fig. 1 *E* and *F*).

Inhibition of CFTR-Dependent Short-Circuit Current by Metformin in MDCK Cells Is AMPK Dependent. We next examined the effect of metformin treatment on the CFTR chloride channel, which is inhibited by AMPK phosphorylation (17–19). Because the CFTR drives, at least in part, the fluid secretion in PKD cystogenesis, we hypothesized that metformin-stimulated AMPK activity would inhibit CFTR channels in renal epithelial cells and slow the rate of cyst growth (20, 21). To test whether metformin inhibits CFTR via AMPK in a kidney-derived epithelial cell line, CFTR was expressed by adenoviral transduction in three different polarized MDCK type II cell lines stably transfected with an empty vector or with shRNA plasmids directed against two isoforms of the catalytic α subunit of AMPK. MDCK cells endogenously express high concentrations of the α1 isoform of the AMPK catalytic α subunit

Author contributions: V.T., K.R.H., and M.J.C. designed research; V.T., S.N., P.S.-M., J.D.K., H.L., and A.K. performed research; L.Z. and S.S. contributed new reagents/analytic tools; V.T., P.S.-M., A.K., K.R.H., and M.J.C. analyzed data; and V.T., K.R.H., and M.J.C. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1011498108/-/DCSupplemental.

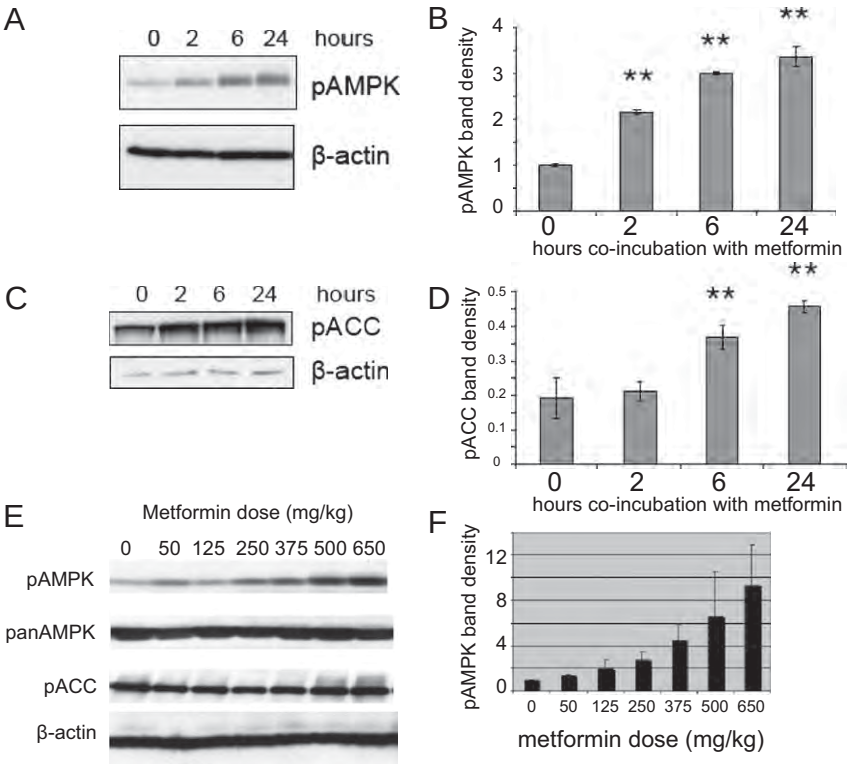

Fig. 1. Metformin activates AMPK in vitro and in vivo. (*A*) MDCK cells were incubated with 1.0 mM metformin for the number of hours stated. Cells lysates were blotted for pAMPK, the activated form of AMPK. (*B*) Quantitation of pAMPK band density normalized to β-actin. Comparisons of the mean (±SEM) are shown for each time point (**P = 0.00002 at 2 h, P = 0.001 at 6 h, P = 0.0005 at 24 h; Tukey's test relative to vehicle-treated control for that set of wells; n = 3 wells for each condition). (*C*) MDCK cells were treated as in *A* and blotted for pACC, a downstream target of pAMPK. (*D*) Comparisons of the mean band density relative to β-actin (±SEM) are shown for each time point. There is no significant change in protein expression between 0 and 2 h (**P = 0.0306 at 6 h, P = 0.005 at 24 h; Tukey's test relative to vehicle-treated control for that set of wells; n = 3 for each condition). (*E*) C57BL/6 mice (8 wk old) were treated i.p. with metformin or with vehicle for 3 d. Western blot analysis of kidney homogenates using anti-pAMPK demonstrates increasing activation of AMPK with increasing metformin dosing. (*F*) Quantitation of Western blot of in vivo pAMPK levels by normalized band density to β-actin. Comparisons of the mean (±SEM) are shown for each time point; n = 3 mice for each dose.

and very low concentrations of the α2 isoform. Expression of the α1 shRNA construct reduced expression of this protein by ∼90%, whereas the α2 shRNA had no effect on α1 protein expression. Knockdown of α1 also reduced the level of total pAMPK by ∼90% (Fig. 2*A*). CFTR-dependent short-circuit current (I_{sc}) was measured for cells grown on filters mounted in Ussing chambers for 4 d following adenoviral transduction, with or without exposure to 1 mM metformin for 2–4 h before measurement. To initiate CFTR-mediated secretion, CFTR-expressing and mock-transduced MDCK cells were treated with the cAMP agonists 3-isobutyl-1-methylxanthine (IBMX) and forskolin, and the experiment was concluded by the application of the specific CFTR inhibitor CFTR-Inh$_{172}$ (22). Typical traces of I_{sc} changes are shown in Fig. 2 *B* and *C*. CFTR-expressing cells generally showed an early peak in I_{sc}, within 1–2 min following forskolin/IBMX treatment, followed by a lower plateau current within ∼5 min. This remaining current was sensitive to inhibition by CFTR-Inh$_{172}$. Metformin (1 mM) pretreatment of empty vector-transfected and AMPK-α2–KD MDCK cells significantly reduced CFTR-dependent I_{sc} by 60–70% relative to cells pretreated with vehicle (Fig. 2*D*). However, there was no metformin-dependent inhibition of CFTR current in AMPK-α1–KD MDCK cells, suggesting that the metformin-induced inhibition of CFTR occurs specifically via an AMPK-α1–dependent mechanism.

Inhibition of mTOR by Metformin in MDCK Cells Is AMPK Dependent. To determine whether metformin induces AMPK-mediated inhibition of mTOR activity, we tested whether mTOR activity is diminished in MDCK cells cultured in the presence of metformin by blotting for the phosphorylated form of the mTOR downstream target ribosomal S6 kinase (S6K) p70 subunit (p70 S6K) (Fig. 3*A*) relative to pan-S6K (Fig. 3*B*). This inhibition is time dependent, with increasing exposure to metformin resulting in greater suppression of this pathway. Total S6K levels remain constant. The inhibition takes longer to achieve than inhibition of CFTR or ACC, consistent with the indirect inhibition of mTOR by AMPK via TSC2/1 and Rheb (Ras homolog enriched in brain) (Fig. 3*C*). This effect is markedly less pronounced in AMPK-α1–KD cells (Fig. S1). To evaluate whether these changes in phospho-protein levels translated into changes in proliferation, an Alamar Blue assay was used to quantitate proliferation in wild-type and AMPK-α1–KD MDCK cells. In figure 3*D*, the *y* axis depicts cell number measured at each given concentration of metformin and normalized to the control value, which was obtained for the same cell type at the same time point without metformin treatment. Wild-type MDCK cells exhibited a metformin dose-dependent decrease in proliferation, but this response was diminished significantly in the AMPK-α1–KD MDCK cells (Fig. 3*D*). At the highest concentration of metformin tested (5 mM), substantial growth suppression was detected in AMPK-KD cells, perhaps because of the low level of residual AMPK that is expressed in these KD cells (Fig. 2*A*) or the effects of high doses of metformin on yet to be identified AMPK-independent pathways. A similar suppressive effect of metformin treatment on proliferation was observed in vivo. We performed immunofluorescence analyses on kidneys from metformin-treated and vehicle-treated cystic *Pkd1*$^{flox/-}$;*Ksp-Cre*

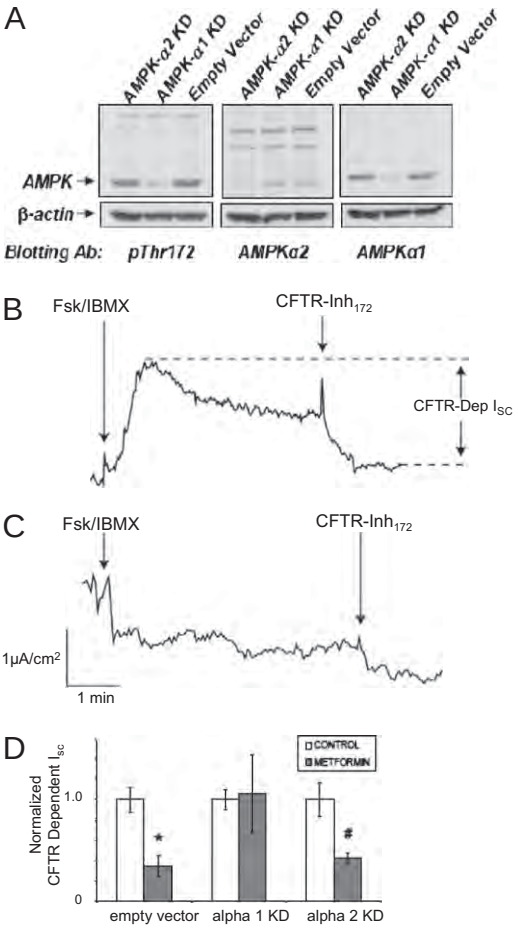

Fig. 2. Metformin inhibits I_{sc} in an AMPK-dependent manner. (*A*) MDCK cells stably expressing empty vector or shRNA plasmids directed against either the catalytic α1 or α2 subunits of AMPK (AMPK-α1–KD and AMPK-α2–KD cells, respectively) were blotted with antibodies against phosphorylated Thr^{172} ($pThr^{172}$), AMPKα2, or AMPKα1 to measure the level of AMPK expression. (*B*) A representative I_{sc} trace of cells with or without 1 mM metformin pretreatment. Mock-transduced or NH_2-terminally GFP-tagged, CFTR-transduced MDCK empty vector control cells, AMPK-α1–KD cells, or AMPK-α2–KD cells were treated with 1 mM metformin or vehicle for 2–4 h before Ussing chamber measurements of I_{sc}. A representative I_{sc} trace of vehicle-pretreated CFTR-expressing empty vector control MDCK cells treated with IBMX and forskolin (Fsk) and then with CFTR-Inh_{172} at the indicated times is shown. (*C*) A similar representative trace of mock-transduced empty vector control cells shows no response to these cAMP agonists or to CFTR-Inh_{172}. There also was no significant change in I_{sc} following addition of 10 μM amiloride, indicating that the epithelial Na^+ channel does not contribute significantly to I_{sc} in these MDCK cells. (*D*) Comparisons of the normalized mean (±SEM) CFTR-dependent I_{sc} in empty vector control, AMPK-α1–KD, and AMPK-α2–KD cells with (dark gray bars) or without (white bars) metformin pretreatment (*P = 0.002, #P = 0.022; unpaired t test relative to vehicle-treated controls for that cell type; n = 6–9 filters for each condition).

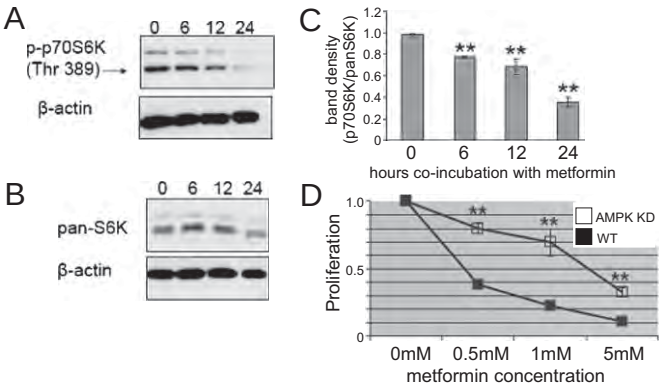

Fig. 3. Metformin inhibits phosphorylation of the mTOR downstream target, p70 S6K, and slows cellular proliferation in an AMPK-dependent manner. A subconfluent monolayer of MDCK cells was incubated with 1.0 mM metformin for the indicated time. Cells lysates were blotted for the downstream marker of mTOR activity. (*A*) p70 S6K. (*B*) Total S6K. (*C*) Quantitation of phospho-S6K Western blot band density normalized to β-actin. Comparisons of the mean (±SEM) are shown for each time point. (**P = 0.00005 at 6 h, P = 0.009 at 12 h, P = 0.00009 at 24 h; one-way ANOVA with Tukey's analysis relative to vehicle-treated control for that set of wells; n = 3 wells for each condition). (*D*) Effect of metformin on proliferation of control MDCK cells and MDCK cells stably transfected with shRNA against AMPK, graphed relative to control. The y axis represents cell number at each concentration of metformin, normalized to the control value measured for the same cell type at the same time point without metformin treatment. (**P = 0.0008 at 0.5 mM, P = 0.009 at 1.0 mM, P = 0.004 at 5 mM; unpaired t tests between both cell lines, comparing rates of cell proliferation with n = 3 per metformin concentration).

(Pkd1, polycystic kidney disease-1 gene; Ksp-Cre, kidney specific cadherin promoter-driven Cre recombinase) mice using an antibody directed against Ki67, a marker of actively proliferating cells (Fig. S2). In kidneys from vehicle-treated mice, 19.7 ± 3.8% of the cells exhibited Ki67 positivity (450 cells were counted from each of six mice) in comparison with 10.6 ± 3.6% of the cells in metformin-treated mice (450 cells were counted from each of four mice) (P < 0.0074). To assess whether the effects of metformin treatment on proliferation correlate with the level of mTOR activity in the cystic kidneys before and after metformin treatment, we performed immunohistochemistry using an antibody directed against the activated form of an mTOR target. As depicted in Fig. S3, we stained tissue from control and metformin-treated cystic mice with an antibody that detects the phosphorylated form of eukaryotic translation initiation factor 4E-binding protein 1 (p4E-BP1), an mTOR target whose level of phosphorylation commonly is used to report levels of mTOR activity (23). We find that the level of p4E-BP1 generally is higher in cyst-lining epithelial cells in control animals than in metformin-treated animals, an observation that is consistent with the interpretation that metformin treatment reduces the level of mTOR activation.

Metformin Treatment Slows Cystogenesis ex Vivo and in Vivo. The 2D culture models do not accurately depict cell growth in the 3D environment in which cysts develop. To evaluate metformin's effects in the context of cystogenesis, we suspended MDCK cells in a 3D collagen matrix and allowed them to form cysts spontaneously in the presence of forskolin and IBMX (24). Cultures coincubated with metformin for the duration of cyst growth produced significantly smaller cysts than those similarly treated with forskolin or IBMX alone (P = 0.003, unpaired t test, n = 3 gels for each experimental condition) (Fig. 4*A*).

We next tested the effect of metformin on ex vivo cystogenesis. Kidneys were removed from C57/B6 mice at embryonic day 12.5 (E12.5). One embryonic kidney was cultured in the presence of membrane-permeable 8-bromo-cAMP (8-Br-cAMP) to stimulate fluid secretion, and the contralateral kidney was coincubated with 8-Br-cAMP and metformin for 4 d. Culture in the presence of 8-Br-cAMP induces cyst formation in embryonic mouse kidneys (4). Metformin treatment significantly decreased the fractional cyst area (P = 0.04, unpaired t test; n = 4 for each experimental condition). On day 5, metformin was removed from the treated embryonic kidney, and cyst growth recommenced in the treated kidney, demonstrating that metformin treatment slowed cyst growth without affecting the viability of the tissue (Fig. 4*B*).

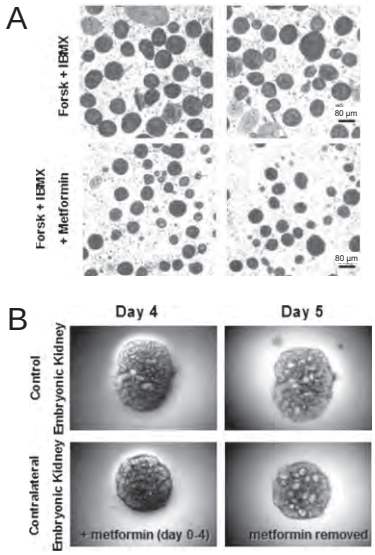

Fig. 4. Metformin reduces cyst size in vitro and ex vivo. (*A*) Representative light micrographs of MDCK cell cysts grown in collagen gels. Cysts were treated with forskolin (Forsk) and IBMX to enhance apical fluid secretion with (*Lower*) or without (*Upper*) 1.0 mM metformin for 20 d. Gels were melted, and the cysts were allowed to precipitate to the bottom for imaging. (*B*) Metformin treatment reduces cyst size in an ex vivo model of renal cystogenesis. Embryonic kidneys were placed in culture at E12 and maintained for 5 d in the continued presence of 100 μM 8-Br-cAMP. Representative light microscopic images from one mouse are shown. Each row shows the same kidney. The contralateral kidney (*Lower*) was treated with metformin for 4 d and then switched to normal medium, illustrating that the embryonic kidney remains viable and capable of cystogenesis.

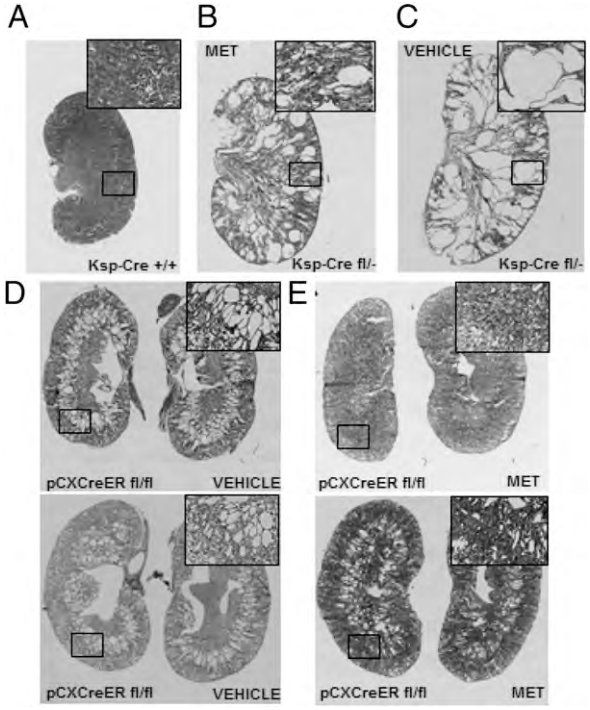

Fig. 5. Metformin treatment reduces the cystic index in two mouse models of ADPKD. (*A–C*) Representative midsagittal sections from the kidneys of (*A*) a *PKD1*$^{+/+}$;*Ksp-Cre* mouse, (*B*) a metformin-treated *PKD1*$^{flox/-}$;*Ksp-Cre* mouse, and (*C*) a vehicle-treated *PKD1*$^{flox/-}$;*Ksp-Cre* mouse at P7. The metformin- and vehicle-treated mice were given daily weight-adjusted i.p. injections from P4 until P6. (*D* and *E*) Representative images from *PKD*$^{flox/-}$;*pCX-CreER* mice treated with vehicle (*D*) or metformin (*E*) from P7–P17, with Cre induction at P9 or P10.

Metformin Treatment Slows Cystogenesis in the in Vivo models of PKD. We next tested whether metformin slows cyst growth in a murine model of PKD. Initially, we used the most aggressive viable murine model of PKD (*Pkd1*$^{flox/-}$;*Ksp-Cre*) in which there is progression of renal cystic disease within the first week of life and death between the second and third weeks of life (6). We treated these mice with daily i.p. injections of metformin (300 mg·kg^{-1}·d^{-1}) dissolved in a 5% (mass/vol) dextrose solution from postnatal day 4 (P4) until P6. This dose is known to activate AMPK (25). Mice then were killed, and kidneys were harvested at P7. The vehicle-treated *Pkd1*$^{flox/-}$;*Ksp-Cre* kidneys (Fig. 5*C*) were profoundly cystic and greatly enlarged compared with the *Pkd1*$^{+/+}$;*Ksp-Cre* kidneys (Fig. 5*A*). In contrast, cyst burden was significantly reduced in the kidneys from the metformin-treated *Pkd1*$^{flox/-}$;*Ksp-Cre* mice (Fig. 5*B*). Because metformin can affect body weight, the kidney weight: body weight ratio was not used as an end point (26). Instead, the effect of metformin on renal morphology was quantitated by evaluating the cystic index, which determines the fraction of a given section that corresponds to luminal area (including both tubule and cyst lumens). Untreated *Pkd1*$^{flox/-}$;*Ksp-Cre* kidneys had a cystic index of 71.4 ± 4.0%, whereas the cystic index of metformin-treated *Pkd1*$^{flox/-}$;*Ksp-Cre* kidneys was 51.8 ± 5.2%. (P = 0.029; unpaired t test; n = 4 control mice and n = 8 metformin-treated mice). In wild-type kidneys, this evaluation calculates a cystic index of 10% resulting from tubular lumens. Notably, although the metformin-treated kidney is still cystic, it displays significantly more parenchyma than the vector-treated control. Although metformin might prevent further cyst growth, it is unlikely that treatment reduces the size of preexisting cysts.

We established an inducible model for Pkd1 inactivation using a conditional *Pkd1*^{flox} allele in combination with a tamoxifen-inducible Cre recombinase (pCX-CreER) (6, 27, 28). Induction of Cre expression before P13 leads to rapidly progressive cystic disease in *Pkd1*$^{flox/flox}$ animals (29). In this system, it is possible to initiate metformin treatment before or during cyst development. Thus, this model might replicate more accurately the clinical scenario in which metformin therapy could commence early in the disease process and act to prevent or slow subsequent cyst development. We initiated metformin treatment (300 mg·kg^{-1}·d^{-1}) at P7 and then injected tamoxifen i.p. at P9 or P10 to initiate disease induction. We continued daily metformin injections until P18, when the animal was killed and kidneys were harvested for histology and cystic index evaluation. Once again, metformin treatment resulted in a smaller fractional cyst burden than seen in vehicle-treated controls (31% vs. 43%; P = 0.041, unpaired t test; n = 6 vehicle-treated mice, and n = 7 for metformin-treated mice), a decrease of nearly one-third in the cyst burden (Fig. 5 *D* and *E*).

Discussion

AMPK activity can be targeted pharmacologically with metformin to reduce the growth of renal cysts. Metformin acts through AMPK to decrease epithelial fluid secretion by directly inhibiting CFTR and to decrease cellular proliferation by indirectly targeting mTOR. Metformin stimulates AMPK phosphorylation in cultured MDCK renal epithelial cells, and this phosphorylation correlates with increased AMPK activity, as evidenced by an increase in the level of the AMPK-mediated inhibitory phosphorylation of ACC. Metformin's inhibitory action on CFTR-mediated chloride transport is AMPK dependent. Additionally, we show that metformin inhibition of mTOR translates into an AMPK-dependent inhibition of cell proliferation. Using both an in vitro model of MDCK cell cystogenesis and embryonic kidneys ex vivo, we demonstrate that metformin decreases cyst size and fractional cyst area. Finally, we illustrate the potential thera-

peutic utility of metformin by testing it in two murine models of ADPKD, both of which are attributable to inactivation of the gene encoding polycystin-1.

Metformin is taken by millions of Americans each year. It currently is approved by the Food and Drug Administration for the treatment of type 2 DM and, intriguingly, for polycystic ovary syndrome, a disease that has a name similar to that of polycystic kidney disease but whose pathogenesis is even less well understood. In fact, metformin often is considered first-line therapy for the treatment of type 2 DM because of its relatively small side-effect profile. Recent literature suggests that metformin's activation of AMPK may be the result of its ability to prevent AMP breakdown, although the exact mechanisms of action of metformin in polycystic ovary syndrome or in type 2 DM remain largely unknown (30). Recent reports also suggest that metformin may exert an antineoplastic effect. It has been reported that metformin acts in a dose-dependent manner to inhibit the proliferation of breast cancer cells, and that this effect can be blocked in the presence of siRNA directed against AMPK (31). This inhibition also is associated with a decrease in mTOR activation, suggesting that metformin's antiproliferative effect is directed through the activation of AMPK and consequent inhibition of mTOR.

In transporting epithelial cells, AMPK not only modulates CFTR activity but also inhibits the epithelial sodium channel (ENaC) (32–34). Although in the cystic kidney this effect conceivably could lead to decreased fluid absorption and therefore perhaps to increased accumulation of cyst fluid, the role of ENaC in cyst-lining epithelial cells is uncertain (35). CFTR can inhibit ENaC channel function directly. Thus, inhibition of CFTR by AMPK could reduce such sodium channel inhibition (36, 37). Taken together, the effects of AMPK activation on ENaC function in the context of renal cystic disease are bimodal and complex. The net effect of AMPK modulation in vivo, however, is likely to reduce luminal fluid accumulation (38).

Numerous therapies for ADPKD, including vasopressin receptor inhibitors, calcium-sensing receptor inhibitors, CFTR inhibitors, cell-cycle inhibitors, and rapamycin, are in development or in clinical trials (4, 9, 39, 40). Each of these strategies targets one of the key processes (proliferation and secretion) thought to be involved in the pathogenesis of PKD. By acting through AMPK, metformin may offer the significant advantage of blocking both processes (Fig. S4). Moreover, metformin already is approved by the Food and Drug Administration and generally is well tolerated. The most serious, albeit rare, side effect of metformin is lactic acidosis and, because metformin is cleared by the kidney, chronic renal disease has been considered a potential predisposing factor for this complication. Ideally, however, metformin use could be initiated at an early stage in ADPKD progression, before the development of substantial cyst burden and compromise of renal function, thus allowing maximal preventive benefit and minimizing the potential for renal dysfunction to limit the safe use of the drug (41, 42). Given the relatively late onset and slow progression of ADPKD, it is conceivable that, even if metformin were to have only modest effects in delaying or slowing cyst development, it might increase significantly the time to the development of end-stage renal disease and perhaps reduce the need for renal replacement therapy.

In this study, only one dose known to activate AMPK in vivo was tested. When considered on a simple milligram per kilogram body weight basis, this dose appears considerably higher than the current maximum dose prescribed for patients with diabetes or polycystic ovary syndrome. However, human-equivalent dose extrapolation is calculated more accurately based on body surface area than on weight. When this calculation is performed for a 60-kg adult, the dose used in our mouse studies extrapolates to a daily dose of ~1,500 mg (43), well within the range in which metformin is safely used in humans. We have not tested the efficacy of lower doses or of alternative dosing regimens in these mouse models. It is likely, however, based on the established pharmacokinetics of metformin, that single daily dosing is suboptimal, and thus we almost certainly did not observe the maximal suppressive effects that metformin potentially could exert on the severity of cyst growth (44). Support for this contention derives from the data presented in Fig. 4*C*, because in the embryonic kidney model, cyst growth resumes rapidly shortly after removal of metformin from the culture medium. Thus, short-term intermittent exposure to metformin may not be adequate to suppress cyst development optimally. It is quite possible that even lower doses administered more frequently might produce beneficial effects in the setting of polycystic kidney disease. It is important to note that our efforts to assess effects of metformin treatment on renal functional parameters such as serum concentrations of serum urea nitrogen and creatinine were inconclusive, in part because of interindividual variability. Further studies, perhaps using more slowly progressive disease models, will be required to reduce this variance and to assess the extent to which metformin treatment can protect or improve renal function in the setting of PKD. In addition, subsequent development of metformin for this clinical application will require pharmacokinetic and pharmacodynamic studies designed to identify an ideal dosing regimen that achieves maximal activation of renal tubular AMPK.

In conclusion, we find that metformin stimulates AMPK, resulting in inhibition of both CFTR and mTOR and thereby both epithelial secretion and proliferation. Our data suggest the possible utility of metformin as a therapy for ADPKD and that AMPK is a potential pharmacological target for ADPKD therapy. The large body of knowledge associated with metformin administration might facilitate the translation of these findings into clinical trials to test the proposition that metformin is a safe and promising approach that exploits AMPK activity to treat this challenging disease.

Methods

Western Blotting and Proliferation Assay. Cultured MDCK cells were lysed, and protein was extracted for Western blotting using standard protocols. For experiments involving AMPK activation in vivo, kidneys were snap-frozen in situ, and homogenates were prepared according to published protocol before Western blotting (45). Details and antibodies used are given in *SI Methods*.

Generation of AMPK-KD Cell Lines. AMPK-KD cell lines were established by lentiviral infection. Further details and targeting sequences are given in *SI Methods*.

CFTR Short-Circuit Current Measurements in MDCK II Cells. MDCK cells expressing either empty vector or shRNA against one of two AMPK isotypes underwent adenoviral transduction to express GFP-tagged CFTR. I_{sc} was assessed by Ussing chamber measurement after stimulation with forskolin and 3-isobutyl-1-methylxanthine and then CFTR-Inh_{172} to determine the CFTR-dependent change in I_{sc}. Cells were pretreated with vector or metformin as noted. Further details are given in *SI Methods*.

In Vitro Cystogenesis. MDCK cells were suspended in a collagen matrix as previously described by Grantham and coworkers (24). Further details and quantitation method are given in *SI Methods*.

Ex Vivo Cystogenesis. Embryonic kidneys were microdissected from timed pregnant C57BL/6 mice at E12.5, cultured per standard protocol with the addition of 8-Br-cAMP to promote cyst formation, and treated with either metformin or vector (4, 46). Further details are given in *SI Methods*.

Mouse Strains, Histology, and Cystic Index. All animal protocols were approved and conducted in accordance with Yale Animal Resources Center and Institutional Animal Care and Use Committee regulations. *Pkd1*$^{flox/flox}$ and *Ksp-Cre* lines have been described previously (6, 47, 48). From P4 until P6, experimental mice received either metformin (300 mg/kg body weight) dissolved in 5% (mass/vol) dextrose or 5% (mass/vol) dextrose alone through daily i.p. injections. These mice were killed at P7. The *pCX-CreER* transgenic line (kindly provided by Corinne Lobe, University of Toronto, Toronto) gives generalized Cre expression based on the pCAGGS chicken β-actin promoter construct. Cre recombinase translocation to the nucleus was induced by

a single dose (0.1 mg tamoxifen/g body weight) given by i.p. injection at P9 or P10 (27, 28). Kidneys were harvested as described in *Results* and fixed, and the fractional cyst area was calculated via MetaMorph (Universal Imaging). Further details are given in *SI Methods*.

ACKNOWLEDGMENTS. We thank SueAnn Mentone and Deren Shao for their expert assistance with specimen preparations, Corinne Lobe for providing the *pCX-CreER* mouse line, the members of the K.R.H. and M.J.C. laboratories for their helpful advice, and Drs. Jennifer Pluznick and Gerhard Giebisch for careful readings of this manuscript. This work was supported by National Institutes of Health Grants MSTP TG 5T32GM07205 and F30DK083221 (to V.T.), T32 HL007563 (to J.D.K.), DK075048 (to K.R.H.), DK54053 and DK51041 (to S.S.), DK57328, and DK17433 and Department of Defense Grant CDMRP PR093488 (to M.J.C.), and by a grant from the Cystic Fibrosis Foundation (to K.R.H.).

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